MORPHOLOGICAL EXAMINATION METHODS IN NEPHROLOGY-DIABETES RELATED RESEARCH AND IN CLINICO-PATHOLOGY

DOCTORAL (Ph.D.) THESES

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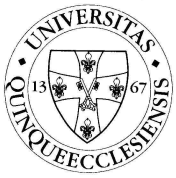

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PÉCS, 2009

LIST OF ABBREVIATIONS

AIN	acute interstitial nephritis
AEC	amino-ethylcarbasol
AGE	advanced glycation endproducts
CLSM	confocal laser-scanning fluorescent microscope
CO	control
DAB	diaminobenzidine
EM	electron microscope
F	fibrillary
F/f	fibrinogen/fibrin
FITC	fluorescein-isothiocyanate
FSGS	focal segmental glomerulosclerosis
GB	glomerular bleeding/ glomerular type red bloood cells
GBM	glomerular basement membrane
GN	glomerulonephritis
GP	glomerulopathy
HBP	high blood pressure
IC	intracellular
IF	immunofluorescence
IGT	impaired glucose tolerance
IH	immunohistology
IT	immunotactoid
LM	light microscopy
MF	microfibrillary
MGO	methylglyoxal
MT	microtubular
OGTT	oral glucose tolerance test
PAS	periodic acid Schiff
RAAS	renin-angiotensin-aldosterone system
RBC	red blood cell
SF	soluble fibrinogen
SOS	specific organized structures
STZ	streptozotocin
TB	Tris buffer
TI	tubulointerstitium
XFb	cross-linked fibrin
VMH	ventromedial hypothalamus

Introduction and aims:

1.) Fibrinogen - fibrin is often detected in various localizations, with different intensities and patterns in human renal biopsy specimens using immunofluorescent (IF) examination in the glomerular and tubulointerstitial parts of the kidney. We will use the expression “fibrinogen-fibrin” in the theses until we present our results, as commercially available, well established antisera react in general both with fibrinogen and fibrin. Presence of fibrinogen-fibrin in the glomeruli or the interstitium is always part of a pathological process, it cannot be observed in intact, healthy renal tissue. In many renal diseases, the presence and pathological role of fibrinogen/fibrin is only partially known, sometimes contradictory or even unknown. The methods used in routine i.e. light microscopy (LM), direct IF and electron microscopy (EM) do not provide answers to these questions, thus we have chosen the method of confocal laser scanning fluorescent microscopy (CLSM). We have introduced a previously unused double labeling method for our study. We have examined whether CLSM is a suitable tool to answer these questions. We also determined what is the exact localization of fibrinogen/fibrin and whether there is a correlation between the deposition of fibrinogen/fibrin and severity of the particular disease, and if there are characteristic patterns for distinct renal diseases?

2.) Hematuria may be a sign of various urogenital diseases, from inherited diseases to inflammations or malignancies. It is a hard differential diagnostic problem to find out the cause and localization of hematuria. In cases of glomerulonephritises (GN) and other glomerular diseases, glomerular type, so-called dysmorphic red blood cells get into the urine, that is characteristic for glomerular bleeding (GB). We have tested whether methylglyoxal – a compound of carbonyl stress – is able to promote the formation of glomerular type red blood cells *in vitro*?

3.) An early phase of carbohydrate disorders is impaired glucose tolerance (IGT). This state is positioned between normal metabolism and diabetes mellitus. In diabetic renal diseases, increased intrarenal renin production and appearance of advanced glycation end-products (AGE) has been described. Diabetic nephropathy may develop in diabetic patients in several years or decades, and in patients with high blood pressure (HBP) nephrosclerosis will develop. Diabetes and HBP often occur in the same patient, and they enhance each others damaging ability. Renal complication in these diseases is not yet well understood, but it is clear that an early therapy can slow down, stop or in an ideal situation even reverse the decline of renal function. Therefore it is of high importance to understand development of renal damage in the earliest phase of these diseases.

IGT and HBP induced in rats has led to renal damage. According to our knowledge, morphological and histomorphological alterations developing in IGT+HBP are not yet known.

In an animal experiment we have examined i) the pathophysiological-homeostatic alterations produced by simultaneously induced IGT and HBP (IGT+HBP), ii) the presumed activation, presence and localization of AGE-imidazolone and renin and iii) development of morphological alterations in the kidney.

4.) On the electronmicroscopic (EM) examination of human renal biopsy specimens, especially in immunopathogenetic glomerulonephritises, we have observed various electrondense deposits in various areas of the renal glomerulus. These deposits may have a homogenous structure on low magnification in electronmicroscopy, or they may have a very fine, even granular electrondense appearence. More rarely we may observe special organized structures of various electrondensity. These structures may be quite diverse and can only be studied using electron microscopy.

The organized structures may have a fibrillary or tubular structure, and the fibrils or tubules may appear either quite unorganized or rather structured, parallel and/or bundle-like, fish-swarm-like. Other deposits may have a structure that resembles that of collagen ("collagen-like") or is equivalent or similar to fibronectin. Organized deposits may refer, in some cases, to hereditary nephropathies.

The knowledge of these special, organized structures is of high importance, as these structures that may appear in the routine electronmicroscopy have a high diagnostic value: they may for instance confirm assumed or probable diagnoses like SLE or cryolobulinaemia. On the other hand, it may provide the diagnosis or the probability of not suspected diseases such as multiple myeloma or lymphoma etc.

In the present study we have focused within the organized structures on the so-called immunotactoid, microtubular-fibrillary glomerulopathies.

Materials and methods:

1.) We have performed light microscopic (LM), immunofluorescent (IF), electron microscopic (EM) and confocal laser-scanning fluorescent microscopic (CLSM) analyses of human renal biopsy materials, with a double labeling at the latter method. We investigated the characteristics of the glomerular deposition of fibrinogen-fibrin in glomerular diseases. We have compared the amount of tubulointerstitial fibrinogen-fibrin in glomerular and tubulointerstitial diseases. For the comparison of measurement data of glomerular versus tubular diseases, independent samples t-test was used in the SPSS software. A p value of <0.05 was considered as statistically significant.

2.) We have incubated the suspension of red blood cells (RBCs) at room temperature with methylglyoxal (MGO). We examined the samples with LM with the condensor lens in the

lowered position, at 400-times magnification. Scanning EM was also carried out to look for deformities of the RBCs. Results of the light microscopic analysis were expressed as mean ± SEM, Kruskal-Wallis test and Mann-Whitney U test were used for statistical evaluation.

3.) We gave intracerebral microinjections of MGO into the ventromedial hypothalamus (VMH) of male Wister rats. 'Oral' glucose tolerance test (OGTT), plasma cholesterol, triglyceride, uric acid, insulin and leptin levels were measured. Blood pressure of the animals was measured in a semiautomatic way. The obtained data were analyzed using analysis of variance (ANOVA) and t-tests. From the kidneys fixed with formaldehyde and embedded into paraffine, immunohistologic (IH) analysis of renin, AGE and endothelium was carried out and also renin was immunoelectronmicroscopically detected using a flat embedding method.

4.) Our 2110 renal biopsy cases were analyzed using IF, LM and EM. A detailed clinico-pathological analysis was undertaken in cases of immunotactoid-microfibrillary-microtubular GP diagnosed between 1999 and 2003.

Results:

1.) Using our method – i.e. CLSM and double labeling – we could accurately localize fibrinogen/fibrin. In the non-crescentic cases, in the background of fibrinogen/fibrin positivity, only fibrinogen (SF) was detected, while fibrin (XFb) was present in none of the cases. The *glomerular* localization of fibrinogen was quite diverse in the investigated renal diseases, but showed a good correlation with the areas where LM- and EM-detectable alterations were present. We calculated the area of *tubulointerstitial* fibrinogen positivity in both the group of glomerular diseases and the group of tubulointerstitial diseases, and

comparing the two groups following results were obtained: there was a significant difference between glomerulopathies and the tubulointertitial diseases in the magnitude of tubulointerstitial SF positivity (487,490± 28,033 A.U. vs. 1,035,733 ± 52,664 A.U., $p<0.001$).

2.) Glomerular type RBCs were formed in the presence of MGO. We observed RBC morphological alterations that are seen in GB. One or more “blebs” were formed on the surface of the RBCs. This effect was time- and concentration-dependent. The percentage of glomerular-type RBCs within the MGO-treated RBC suspension at 30 min incubation time increased with increasing concentrations of MGO, it was 5.4 ± 3.9 % in the control state, while the percent of damaged RBCs inceased to 21.9 ± 8.6 % due to 0.25 mM MGO, to 29.3 ± 5.8 % due to 0.5 mM MGO and to 48.9 ± 4,8 % due to 1 mM MGO (all $p < 0.01$ vs. control). The effect of 1 mM was already well-established at 10 min incubation time (percentage of glomerular-type RBCs: 38.1 ± 4.1 %; $p < 0.01$ vs. control) and the effect of 20 min incubation (percentage of glomerular-type RBCs: 49.6 ± 4.4 %; $p < 0.01$ vs. control) was similar to that of the 30 min incubation.

3.) In the MGO-treated rats, IGT and HBP have developed. We could observe renin- and imidazolone positivity in the mesangial area of the glomeruli as well as in the papillary area of the kidney according to the peritubular capillaries, and fibrosis has developed in the same area i.e. the papillary part of the kidney.

4.) We worked out a new algorithm for the differential diagnosis of so-called immunotactoid, microtubular-fibrillary glomerulopathies presenting with organized EM deposits.

The clinico-pathological significance of fibrillary, microtubular and immunotactoid glomerulopathies is as follows:

1. It can be only diagnosed using electron microscopy.
2. Most patients undergo a renal biopsy because of symptoms of a renal disease. In the background of fibrillary and mainly in immunotactoid glomerulopathies we may find a clinically undiagnosed disease such as malignant or benignant lymphoproliferative disease CLL etc. Therefore, targeted clinical tests have to be carried out to reveal the underlying disease. Due to the early and "by chance" diagnosis, the underlying disease as well as the renal disease may be cured and/or the progression may be slowed down.
3. It may indicate the renal manifestation of one, in general malignant, often hematological disease such as CLL. The renal disease can in this case only be cured by the therapy of the underlying disease (e.g. the CLL).
4. In some cases, when only early and/or uncertain clinical-laboratory observations are present, detection of the organized deposits may complete the diagnosis, this way providing an indication for the until then untreated disease, such as in monoclonal gammopathy of unknown significance (MGUS).

Discussion:

1.) Based on our results, we can say, that in our cases we could detect the presence of soluble fibrinogen (SF) in the renal glomeruli and tubulointerstitial areas. Cross-linked fibrin (XFb) was present on no cases except for the RPGN positive controls. In publications on renal diseases, many authors write about detection of fibrinogen or fibrin, in part consequently in title and text, and in part inconsequently. Most authors have used DAKO products. However, the DAKO-produced polyclonal, rabbit anti-human fibrinogen antibody cross-reacts with native fibrinogen (SF), D and E fragments of fibrinogen and fibrin, as well. Thus, those authors, who have not used another differential diagnostic tool, could not be sure whether they detected fibrinogen or fibrin. Therefore, in our study, we also performed Mallory's

phospho-tungstic acid hematoxylin staining of formaldehyde-fixed paraffin-embedded sections besides the IF examination using the DAKO antibody in all cases. This staining serves the detection of XFb. Besides that, we also performed EM examinations, where XFb appears as a special, organized fibrillary structure. The combined use of the three methods makes it possible to distinguish well between SF and XFb. Some workgroups have used anti-human-D-dimer to detect fibrin, others have used this method to detect fibrinolysis either with or without pre-treatment with plasminogen. D-dimer is a fibrin degradation product. In one study on 25 patients with IgA nephropathy and 12 patients with Henoch-Schönlein's purpure, the authors used anti-human fibrinogen to detect fibrinogen-fibrin-related antigen (FRA) and also XFb was investigated, the latter using anti-D-dimer and prior to and after treatment with plasmin. In cases of intense FRA deposition, localization of XFb after treatment with plasmin resembled more the distribution of FRA, than it did prior to treatment with plasmin. The question can be raised, whether D-dimer positivity indicates fibrin or is rather a marker of fibrinolysis? It is hard to explain, that similar results were obtained in one study without plasmin pre-treatment and in the other study with and without plasmin pre-treatment. We would like to emphasize that we can much more accurately characterize the localization of fibrinogen as compared to previous publications. In the literature, deposition "in the capillary wall" was described. However, the capillary wall envolves the layer of the endothelium as well as that of the glomerular basement membrane (GBM). Using our method, we could determine whether fibrinogen is localized only in the endothelium, only in the GBM or in both structures.

The intact unit of peritubular capillary – tubular epithelium represents the "Achilles-heel" of the kidney. In many cases, interstitial fibrinogen positivity observed in our tubulointerstitial renal disease cases may represent extravasation indicating the disrupture of the connection of peritubuar capillary and tubular epithelium.

2.) According to our data, in vitro, in the presence of MGO characteristic morphologic alterations develop on RBCs, that are similar to those seen in GB or glomerular hematuria. These GBs (= dysmorphic RBCs) show specific bleb formation (one, two or more) that can be easily observed in unstained urine sediment. These GBs resemble – according to some authors – the Mickey mouse figure known from comics of Walt Disney. These RBC alterations can be observed in glomerulonephritises with immunopathological background, but also in other non-glomerulonephritic glomerular diseases such as Alport's syndrome in the urine sediment. Knowing these RBC alterations is important in the differential diagnosis of renal diseases, as their presence indicates glomerular renal disease.

According to previous ideas, blebs and thus GBs are either formed in the renal tubuli as a consequence of osmotic stress, or during the passage of RBCs through the GBM. However, it has also been shown that osmotic stress alone is not enough to provoke the formation of GBs. It is questionable whether passing through the GBM alone is sufficient to provoke formation of GBs, as the use of loop diuretics influences RBC morphology in the urine.

We can detect GBs also in Alport's syndrome and in thin basement membrane syndrome. The question may be raised, whether inflammation-induced increased carbonyl stress is present in these diseases, and if so, how it develops. According to novel studies, inflammation may be present in Alport's syndrome, too.

According to our results, MGO has induced oxidative stress and intracellular calcium accumulation in isolated human RBCs. These effects could be prevented using antioxidants. As a consequence of the toxicity of MGO, dysmorphic RBCs were formed, similar to those seen in glomerular hematuria. The formation of GBs is concentration-dependent, and is already present after 10 minutes of incubation. One possible mechanism of bleb formation of RBCs may be carbonyl stress.

3.) In our animal study, impaired glucose tolerance (IGT) and high blood pressure (HBP) developed in MGO-treated rats. In these animals, we observed renin and imidazolone positivity in the mesangial area of the glomeruli, as well as in the peritubular capillaries of renal papillae, and fibrosis has developed in the latter region. It is known that tubulointerstitial damage and consequent interstitial fibrosis plays a crucial role in various renal diseases, and is a determinant of the progression of the disease. It is known that activation of the intrarenal renin-angiotensin-aldosterone system (RAAS) and the accumulation of AGEs plays a role in the development of tubulointerstitial disease/damage. Increased renin mRNA levels have been described in proximal tubular cells of patients with diabetic nephropathy and in rats with streptozotocin-induced (STZ) diabetes. Renin was detected in proximal tubular cells of STZ rats and in transgenic (mREN-2)27 rats with STZ-diabetes. Renin expression has also been described in the proximal tubular epithelilal cells in animals and in humans. According to our knowledge, we were first to describe the induction of renin in the endothelial cells of the peritubular capillaries in the renal cortex.

Non-enzymatic glycation is another important pathogenetic factor in renal diseases connected to disorders of the carbohydrate metabolism. AGE accumulation was described in proximal tubular cells of STZ-induced diabetic rats and expression of imidazolone in renal tubular cells of diabetic patients. According to our knowledge, we are first to describe the presence of AGE-imidazolone in the endothelial cells of the peritubular capillaries in the renal papillae of rats with IGT and HBP.

The pathomechanism of the induction of renin and imidazolone in the peritubular capillary endothelial cells is not known. One possible mechanism is that they get from the tubular epithelial cells into the peritubular capillary endothel cells, or it is *de novo* synthesized in the

endothelial cells, and/or it gets into the circulation from the juxtaglomerular apparatus also via recapillarisation. Clarification of the pathomechanisms requires further studies.

The exact pathomechanism of tubulointerstitial (TI) fibrosis is not known. We have verified accumulation of renin and AGE in the tubulointerstitium of renal papillae of MGO-treated rats, and fibrosis developed in the same area. The RAAS is locally very active in the kidney. Infusion of angiotensin II leads to inflammatory cell infiltration of the tubulointerstitium and may lead to glomerular and interstitial fibrosis in rats. Angiotensin II enhances proliferation rate in renal fibroblast cell culture, and increases the expression of fibronectin, collagen and transforming growth factor-β (TGF-β). TGF-β is the most potent profibrotic cytokine. Upon these data we can assume that the accumulation and co-localization of renin and imidazolone may play a role in the development of tubulointerstitial fibrosis in the renal papillae.

4.) In various renal diseases, microfibrils and microtubuli can be observed ultrastructurally in the mesangium and the glomerular basement membrane, we can call these states glomerulopathies with organized deposits. Microfibrils do not have a lumen, they show a random structure and are composed of amyloid proteins, immunoglobulins or extracellular matrix proteins. Microtubuli do have a lumen, they are generally organized into parallel bundles and are composed of immunoglobulins.

Some researches distinguish between fibrillary glomerulonephritis (FGN) and immunotactoid glomerulopathy (ITG) upon structure of the deposits. Others regard all states with Congo-red negative immunoglobulin depositum a single disease with different ultrastructural morphology (“ITG”). A further, third group uses the expression “fibrillary-immunotactoid glomerulopathy”. Thus there is no uniform clinico-pathological classification of FGN-ITG.

We have examined the properties of FGN and ITG separately. FGN is characterized by the deposition of randomly structured, elonged, non-branched microfibrils in the mesangium and

the GBM. Diameter of the fibrils is approx. twice of the amiloid fibrils, between 15-30 nm, most of them are ~ 20 nm thick. Clinical presentation of the renal disease is subnephrotic or nephrotic range proteinuria. Proteinuria is often accompanied by macro- or microhematuria, high blood pressure and renal failure.

ITG is characterized by microtubular deposits that have an outer diameter of 10-90 nm, the center of them is empty, electro-lucent and they are organized into parallel rows in the GBM and the mesangium. ITG is a rare entity, clinical presentation of the ITG is similar to that of FGN, however in ITG patients we can often observe the co-incidence or the later development of monoclonal gammopathy or lymphoproliferative diseases (mainly B-cell lymphocytic leukaemia or small lymphocytic non-Hodgkin lymphoma) and hypocomplementaemia. These facts give a high clinico-pathological importance to FGN-ITG.

THESES

1.1 We were able to detect soluble fibrinogen in the glomeruli and in the tubulointerstitial area in non-crescentic glomerulonephritises and tubulointerstitial nephritises. Cross-linked fibrin could be detected in none of the cases.

1.2 We were able to localize fibrinogen in a much more accurate manner compared with previous publications using a double labeling and confocal laser scanning microscopy.

2. Methylglyoxal may lead to the formation of dysmorphic red blood cells, similar to those seen in glomerular-type hematuria.

3. We were first to demonstrate the presence of renin and imidazolone in the peritubular epithelial cells of male Wistar rats that developed IGT and high blood pressure.

4. We worked out an improved algorithm for the differential diagnosis of so-called immunotactoid, microtubular, fibrillary glomerulopathies presenting with organized electronmicroscopic deposits.

IF data of the author:

Total IF including abstracts:	***98.304***
Total IF excluding abstracts:	***19.883***
IF of publications on which the theses were based:	***20.717***
IF of publications on which the theses were based excluding abstracts:	***4.277***

PUBLICATIONS ON WHICH THE THESES WERE BASED

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